Biocontrol Strategy Development for Lettuce Drop Disease Management

This project was completed on June 30, 2009

Project Summary

Lettuce drop caused by two related fungi, *Sclerotinia sclerotiorum* and *S. minor*, is one of the most important fungal diseases affecting lettuce production in Arizona. As such, considerable effort is directed towards disease management and yield loss prevention. Current management strategies rely heavily on chemical applications with moderate success. Novel strategies are needed to 1) provide improved disease suppression, 2) supplement current chemical strategies to prolong their efficiencies, and 3) reduce environmental impacts. Biocontrol is one such strategy**.** Of the biocontrol organisms tested against *Sclerotinia*, *Coniothyrium minitans* is promising and has consistently performed well against *S. sclerotiorum.* In contrast, *C. minitans* has not performing well against *S. minor* at standard application rates. However, results from recent trials revealed that very high application rates of Contans completely prevented disease development caused by *S. minor*. The application rate used in that study was 5X the recommended rate. Therefore, this proposed study aims to optimize the application rate and timing of Contans in the development of effective, yet economical biocontrol of lettuce drop caused by *S. minor*. In addition, factor(s) that diffused from *Sclerotinia* that attract and initiate *C. minitans* response will also be identified to better understand biocontrol activity of *C. minitans.*

Project Approach

The main goal of this project was to develop an effective biocontrol strategy for the control of lettuce drop disease caused by *Sclerotinia minor*. The specific objectives were:

1. Optimize the application rate of the biocontol product Contans™ (*Coniothyrium minitans*) to effectively control lettuce drop disease caused by *S. minor* in lettuce production field.
2. Characterize the diffusible stimulating factor (s) from sclerotia of *Sclerotinia* spp. that may elicit *Coniothyrium minitans* response.

Objective 1. A field trial was set up and initiated as described in the project proposal during the 1st quarter of the project (Nov 07). During January, a third application of biocontrol products and chemical fungicide was applied to specific treatment as per described protocol. As with the first and second applications of the biocontrol product, irrigation was performed immediately afterwards to insure maximum survival of biological products in the field. Irrigation and fertilization was continued as needed and consistent with standard cultural practices for lettuce in the Yuma area until the completion of the trial (harvest) that was conducted on 4/7/08. At harvest, all healthy heads were recorded for all treatment plots. Analysis of variance of plot data was conducted to determine significant effects of select treatments on disease incidence.

A second trial was conducted during the winter of 2008-09 as a repeat of the 2007-08 trial to confirm findings and subject data to more robust statistical analysis. These combined data are presented in **Figure 1**.

Objective 2. Exudates were obtained from live *Sclerotinia* sclerotia by soaking sclerotia in sterilized water (1g/10 ml of water) for 1 hr and filtering through 0.2um filter. Collected

exudates were separated into polar and non-polar portions by ethyl acetate fractionation. The aqueous phase containing polar compounds was collected in sterilized tubes and subjected to a preliminary assessment of stimulation activity using a spore germination stimulation assay. A suspension of *Coniothyrium minitans* spores (the biocontrol agent) were mixed with equal volume of fractionated polar compounds in cavity glass slides and incubated at 20°C for 48 hrs.

The percent spore germination was calculated by counting the number of germinated spores in 100 spores on three different slide preparations using a compound microscope (**Table 1**). Thus, the percent of spore germination was the average of 300 spores. Spores mixed with water served as a control.

In addition, the soils were collected from the lettuce production fields and tested, if there is any stimulatory compounds present naturally in soil for *C. minitans* spore germination. Twenty grams of soil was added in 15 ml of sterilized water and mixed well. The extracts were collected in sterilized tubes and stored at 5°C. The spore germination experiment was carried out as mentioned above.

Objective 3:
In addition to the fractionation experiment, another experiment was initiated simultaneously to address the role of lectin in induction of pathogenicity genes in *Coniothyrium minitans* during the parasitism on sclerotia of *Sclerotinia* spp. It has been shown that *C. minitans* successfully parasitizes the sclerotia of ascomycota fungi (*Sclerotinia* spp., and *Botrytis*) but not the sclerotia of basidiomycota fungi (*Rhizoctonia* sp) under laboratory condition. The reason for the ineffectiveness of *C. minitans* against sclerotia of basidiomycota is not known. However, recently it has been shown that fungi belonging to fungal family Sclerotineacea (e.g., *Sclerotinia* spp. and *Botrytis* sp) possess a novel type of lectin with no known homology with any other fungal lectin. Lectins are proteins with sugar binding affinity and play an important role parasitism in other mycoparasitic interactions such as *Trichoderma (a well known biocontrol agent)* pathogenicity on *Rhizoctonia solani* (pathogen) through recognition of and adhesion to their carbohydrate receptors. Thus, it can be hypothesized that difference in lectins present in these two phyla of fungi is the reason for effectiveness of *C. mintians* on Ascomycota fungi (*Sclerotinia*) but not on Basidiomycota fungi.

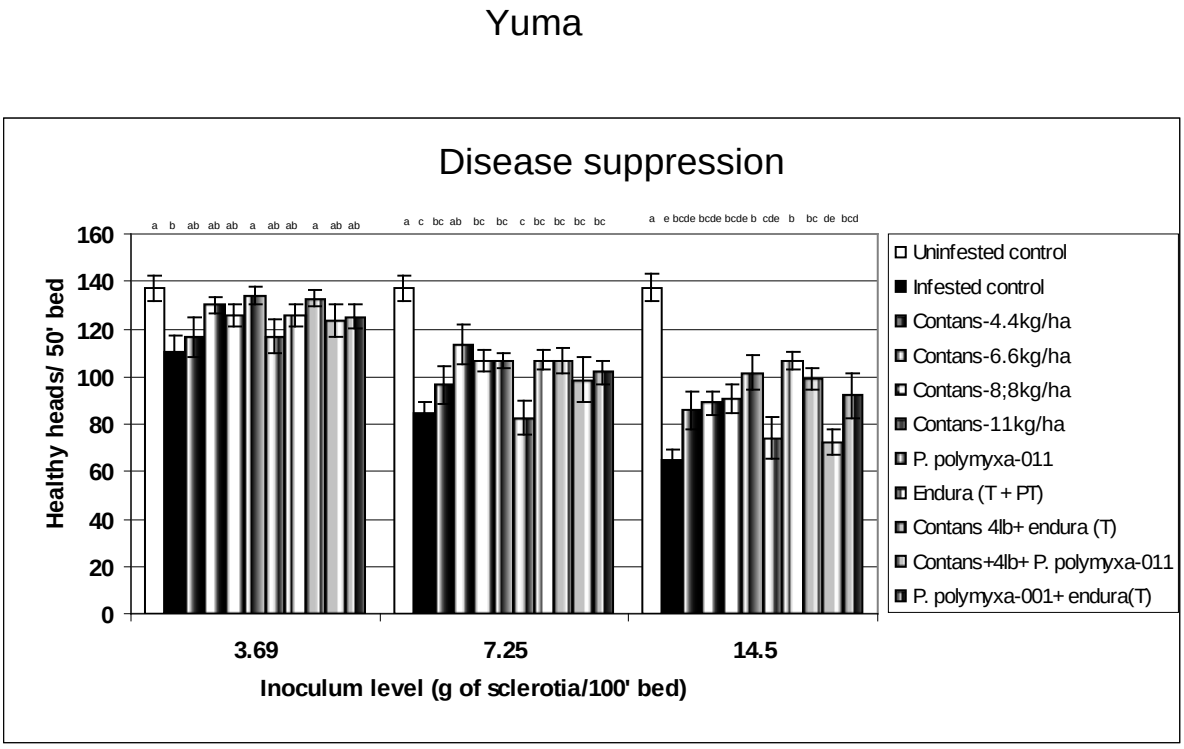
The Graduate Research Associate (PhD student), Ravi Periasamy was recruited 1st quarter to perform most activities related to this project. Two undergraduate students assisted Mr. Periasamy in the execution of the field trials during the 2nd quarter of the project. All personnel were active in the project the entire funding period.

Goals and Outcomes Achieved

Objective 1. Results revealed significant increases of disease in control plots as disease inoculum levels increased from 3.69 g to 14.5 g (19% disease vs 52% disease, respectively). At low inoculum rates, there were no significant differences between 6, 8, or 10 lbs Contans/acre, and all controlled disease nearly 100%. At medium inoculum levels, there were no significant differences in disease incidence between 6, 8, and 10 lbs/acre of Contans, with 6 lbs rate performing best. At high inoculum levels, there was no significant difference between 6 and 8 lbs rates, but the 10 lbs rate performing significantly better. There were no significant

differences between disease incidence in the biocontrol plots and the plots in which the fungicide Endura was used. 4 lbs/acre of Contans resulted in significantly less control than that at higher rates and results were no different than the untreated controls. Thus, results reveal that 6 lbs/acres Contans was just as effective as 10 lbs/acre at medium inoculum levels, and significantly more effective than 4 lbs/acre. These findings are very promising and suggest that much lower rates of the biocontrol product can be used than previously thought for disease control as effective as standard chemical fungicides.

Fig. 1.



Objective 2.

In comparing data from inhibition assay using native sclerotial exudates (unfractionated) and the polar fractions, results revealed that the polar compounds from the sclerotial exudates possessed nearly the same stimulatory potential as did the native exudates. There were no statistically significant differences between exudates from *S. minor* or *S. sclerotiorum* in their capacity to stimulate C. minitans spore germination. Thus, the reason for the effectiveness of biocontrol fungus against *S. sclerotiorum* and ineffectiveness against *S. minor* is likely not due to the differences in quality of sclerotial exudates of this two *Sclerotinia* spp., but may be due to the differences in exudates quantity. Interestingly, soil fractions did not induce spore germination which indicates that germination of *C. minitans* spores mainly depends on the nutrients available from hosts of the fungus, for example, exudes from sclerotia of *Sclerotinia* spp.

To determine the stimulatory properties of non-polar compounds from sclerotia, the same experiment was initiated with little modification. As the non-polar compounds cannot dissolve in water, both polar and non-polar compounds from sclerotia of *Sclerotinia* were collected by soaking sclerotia in sterilized water mixed with ethyl acetate (1g/10 ml of water mixed with ethyl

acetate) for 1 hr and filtering through 0.2um filter. These exudates were separated further into polar and non-polar portions by ethyl acetate fractionation. The non-polar fractions were collected in ethyl acetate, were dried and collected in DMSO. Testing of stimulatory activity of these non-polar compounds is currently running and will be completed over the next few weeks.

Table 1.

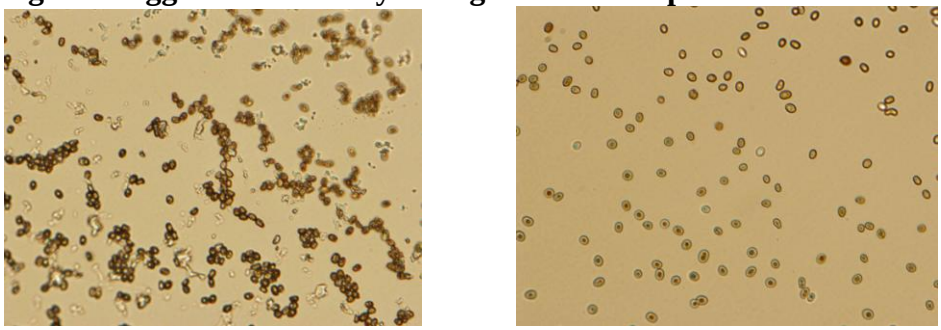
Percent of spore germination*					
Crude exudates		**Polar fraction**		**Soil extract**	**Control (Water)**
S. sclerotiorum	*S. minor*	*S. sclerotiorum*	*S. minor*		
91	95	80	86	0	0

* Values are the average of three replications

Objective 3.

Spore agglutination studies and inhibition assays were successfully carried out to determine the lectin-binding properties of *C. minitans* and the possible role of lectin during the pathogen-biocontrol interaction (Fig. 2). In agglutination studies, *C. minitans* spores were mixed with known quantities of a variety of lectins to determine if sugar-lectin binding would occur resulting in agglutination of spores into masses. Initially, commercially available plant lectins were utilized for this assay. Results from this assay revealed that *C. minitans* spores surface possess sugars specific to a number of lectins used in this study and induced agglutination. However, the incubation times required for agglutination varies with different lectins. Concanavalin A induced agglutination in an hour of incubation which is faster than the other lectins. Both soybean agglutinin and wheat germ agglutinin required 6 hrs of incubation to induce agglutination. However, the agglutination was observed only after 24 hrs of incubation with horse gram lectin.

Figure2. Agglutination assays using *C. minitans* spores



With lectin **without lectin**

There was no agglutination observed in control where spores are incubated with PBS (Table 2). Further agglutination studies using crude extracts from *Sclerotinia* sclerotia also resulted in agglutination, revealing that *Coniothyrium* can bind to lectins in fungal sclerotia. Further studies are underway to obtain highly purified lectins from sclerotia to definitively prove that it is unique sclerotia lectins that are responsible for the observed agglutination of *C. minitans* spores. Interestingly, lectin isolated from jack bean (ConA) inhibited *C. minitans* spore germination significantly suggesting that there may be additional outcomes resulting from spore-lectin interactions. Together, these results revealed that there are clearly interactions between lectins

and surface sugar moities of the biocontrol fungi Coniothyrium, and these interactions during the parasitism may be positive (binding of biocontrol agent and host) or even negative (inhibition of germination of biocontrol agent).

Spore germination assay showed that sclerotial exudates of both *S. sclerotiorum* and *S. minor* induced spore germination in 48 hrs of incubation and the percent spore germination was 87 and 96 % for *S. sclerotiorum* and *S. minor*, respectively. However, the percent spore germination with both sclerotial exudates of Sclerotinia spp., significantly decreased in the presence of Concanavalin A and it was 2.1 and 3.1 % with sclerotial exudates of *S. sclerotiorum* and *S. minor*, respectively. The percent spore germination with either of the *Sclerotinia* exudates decreased with the presence of either, SSA, WGA, or horse gram lectin. Spores mixed with either lectin or PBS alone did not germinate (Table 3 and 4).

Table 2. Agglutination efficiency of *Coniothyrium minitans* spores with different lectins

Lectins	Time required for agglutination (hrs)
Concanavalin A	**1**
Soybean agglutinin	**6**
Wheat germ agglutinin	**6**
Horse gram lectin	**24**
PBS (control)	**No agglutination**

Table 3. Percent spore germination of *C. minitans* in sclerotial exudates of *Sclerotinia sclerotiorum* in the presence and absence of lectins

Treatments	Percent spore germinationA*
Spores + sclerotial exudates + ConA	**21c**
Spores + sclerotial exudates + SSA	**72.1ab**
Spores + sclerotial exudates + Horse gram	**87a**
Spores + sclerotial exudates + Coral tree	**69b**
Positive control	
Spores + SS exudates	**87a**
Spores + lectins	**0**
Spores + PBS	**0**

*** Average of three replications and two repetitions, ConA-Concanavalin A, SSA-soybean agglutinin, WGA-wheat germ agglutinin**
Columns with different letters are significantly different according to Tukey test (P<0.05) based on the F test in analysis of variance

Table 4. Percent spore germination of *C. minitans* in sclerotial exudates of *Sclerotinia minor* in the presence and absence of lectins

Treatments	Percent spore germination*
Spores + sclerotial exudates + ConA	**3.1b**
Spores + sclerotial exudates + SSA	**93.2a**
Spores + sclerotial exudates + Horse gram	**94a**
Spores + sclerotial exudates + Coral tree	**97a**
control	
Spores + SM exudates	**96a**

Treatments	Percent spore germination*
Spores + lectins	**0**
Spores + PBS	**0**

Expected Measurable Outcomes:
Measurable goals for outreach include 1) outreach to 500 researchers, industry professionals, and growers through results posted on extension website, 2) outreach to 500 researchers, industry professionals, and growers through distribution of extension fact sheets, and 3) outreach to 100 researchers, industry professionals, and growers at the annual Southwest Ag Summit. Regarding goal 1), the website "Lettuce Disease Management In Arizona" has been created (http://cals.arizona.edu/plp/sclerotinia/index.htm), password protection (user = sclerotinia, password = online). Public posting of the website was slated for November 2009, but has been delayed because of industry input requesting a more user friendly interface for website navigation. We are currently in the process of redesigning the navigation buttons and this should be complete by early 2011. Our measurable outreach of reaching 500 researchers, industry professional, and growers will easily be exceeded once the site is posted. Regarding goal 2), a comprehensive data fact sheet has been developed specifically focused on disease management of lettuce drop and is ready for distribution. A limited number of these have been printed and are available for distribution. However, following discussion with industry representatives it has been decided that the most optimal means for dissemination of this fact sheet is through a downloadable version on the Lettuce Disease Management In Arizona website. A button directing the downloading of this fact sheet has been added to the website slated for release in January. Again, following posting of the website, the measured goal of reaching 500 clients with our fact sheet will easily be exceeded with a downloadable vs. printed fact sheet. Regarding goal 3), despite requests to be included in the Southwest Ag Summit, the agendas are dictated and filled according to the priorities established by the planning committee. As such, other critical issues in cultivar breeding, cultural practices, and food safety issues have recently risen to a higher immediate priority for the lettuce industry and are included in the limited agenda of the Summit. However, Summit organizers repeatedly assured me that the concern over lettuce drop has not abated and they plan to include a session on the management of this disease in either of the next two planned summits. They have assured me I will be contacted once this inclusion has been finalized.

Beneficiaries

This research will directly translate into improved disease management strategies, which, if enacted, could potentially result in increased productivity for lettuce producers in Arizona and an overall increased competitiveness of the winter lettuce industry in southern Arizona. More broadly, this research will advance agriculture in desert areas, and elucidate at least one mechanism by which many biocontrol strategies fail upon moving laboratory findings to field applications.

Lessons Learned

A no-cost extension was requested by the grantee and approved by ADA on November 13, 2008. The new expiration date for this project was June 30, 2009. Below is an excerpt from the grantee's request for the no-cost extension.

Aspects of the exudates fractionation are challenging and require additional time for completion.

Timetable:
The proposed project will begin in November 2007 and will continue for one year plus 8 months. The field trials will begin with lettuce planting in mid-November and continue until early March 2008 or until crop maturity. Following disease assessment and harvest for head weight, data analysis will be conducted and continue for 1-2 months. The experiments to characterize exudates from sclerotia will begin in August 2008 and will be completed in about 7 months. During this period, the exudates from both *Sclerotinia* spp will be collected and fractionated by HPLC. Then these fractions will be tested individually and in combination for the stimulatory effect on mycelial growth under laboratory conditions. The compound(s) that specifically present in both *Sclerotinia* spp and stimulate the *C. minitans* will be identified at the end of these experiments and will be further characterized by chemical analyses. Data analysis and comparative studies will be conducted over a two month period and be completed by April 2009. Manuscript preparation for both studies will be conducted during May and June 2009.

The execution of this project provided additional training for undergraduate and graduate students in field trial design and setup in desert production areas. Moreover, these trials provided exceptional opportunities for in depth training in the use of a number of statistical analysis programs currently available for field trial applications.

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